APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1993, p. 2546–2551
0099-2240/93/082546-06$02.00/0

Vol. 59, No. 8

Effects of Hydrogen and Formate on the Degradation of Propionate and Butyrate in Thermophilic Granules from an Upflow Anaerobic Sludge Blanket Reactor

JENS E. SCHMIDT AND BIRGITTE K. AHRING*

Anaerobic Microbiology/Biotechnology Research Group, Department of Biotechnology, Building 223, Technical University of Denmark, DK-2800 Lyngby, Denmark

Received 28 December 1992/Accepted 29 May 1993

Degradation of propionate and butyrate in whole and disintegrated granules from a thermophilic (55°C) upflow anaerobic sludge blanket reactor fed with acetate, propionate, and butyrate as substrates was examined. The propionate and butyrate degradation rates in whole granules were 1.16 and 4.0 μmol/min/g of volatile solids, respectively, and the rates decreased 35 and 25%, respectively, after disintegration of the granules. The effect of adding different hydrogen-oxidizing bacteria (both sulfate reducers and methanogens), some of which used formate in addition to hydrogen, to disintegrated granules was tested. Addition of either *Methanobacterium thermoautotrophicum* ΔH, a hydrogen-utilizing methanogen that does not use formate, or *Methanobacterium* sp. strain CB12, a hydrogen- and formate-utilizing methanogen, to disintegrated granules increased the degradation rate of both propionate and butyrate. Furthermore, addition of a thermophilic sulfate-reducing bacterium (a *Desulfotomaculum* sp. isolated in our laboratory) to disintegrated granules improved the degradation of both substrates even more than the addition of methanogens. By monitoring the hydrogen partial pressure in the cultures, a correlation between the hydrogen partial pressure and the degradation rate of propionate and butyrate was observed, showing a decrease in the degradation rate with increased hydrogen partial pressure. No significant differences in the stimulation of the degradation rates were observed when the disintegrated granules were supplied with methanogens that utilized hydrogen only or hydrogen and formate. This indicated that interspecies formate transfer was not important for stimulation of propionate and butyrate degradation.

During methanogenesis from complex organic matter in the absence of electron acceptors other than CO_2, two-thirds of the CH_4 is formed by aceticlastic methanogens (26). CO_2 reduction to CH_4 accounts for almost all of the remaining methane formation. In addition to the quantitative importance of hydrogenotrophic methanogens, the activity of these organisms is essential for proper functioning of other groups of bacteria, especially the obligate proton-reducing acetogens (11). Propionate and butyrate are important intermediates in the anaerobic degradation of complex matter.

The Gibbs free energies ($\Delta G'$) of some of the reactions involved in the oxidation of butyrate and propionate under standard conditions are given in Table 1. Because of the unfavorable energetics, oxidation of propionate and butyrate is only possible if products are removed efficiently by the methanogens, resulting in a low hydrogen partial pressure (29). An H_2 partial pressure below ca. 10^{-4} atm (1 atm = 101.29 kPa) and 10^{-3} atm is necessary for degradation of propionate and butyrate, respectively (2, 7, 14, 18). Such low hydrogen partial pressures in methanogenic systems are achieved by interspecies transfer of molecular hydrogen from hydrogen-producing bacteria to hydrogen-oxidizing methanogens (7, 22, 34). In addition to interspecies hydrogen transfer, interspecies formate transfer has been proposed to play a role in the syntrophic oxidation of fatty acids (5, 30, 32). From the data in Table 1, it can be concluded that in systems oxidating propionate or butyrate, the H_2 partial pressures and formate concentrations must be higher at thermophilic than at mesophilic temperatures in order to achieve the same $\Delta G'$ values.

The development and growth of granular sludge is important for high-rate anaerobic systems such as upflow anaerobic sludge blanket (UASB) reactors (16). The methanogenic granules are produced during self-immobilization of the different metabolic groups into bacterial aggregates (12, 13). The exact composition of the bacterial aggregates depends on the characteristics of the wastewater and on the inoculum used. The high bacterial cell densities in the granules minimize the distances between bacteria and maximize interspecies transfer of acetate, formate, and hydrogen between syntrophic fatty acid degraders and methanogens (5, 9, 21, 30, 31). Granular sludge is therefore a suitable system for studies of the degradation of volatile fatty acids (VFA) because the granule structure gives ideal conditions for syntrophic associations of H_2-producing acetogenic bacteria with methanogens.

In this article, we present data on the degradation of propionate and butyrate in intact and disintegrated granules from a thermophilic (55°C) UASB reactor fed acetate, propionate, and butyrate as substrates. The effect of addition of different hydrogen- and/or formate-utilizing bacteria to disintegrated granules was examined.

MATERIALS AND METHODS

Organisms. *Methanobacterium thermoautotrophicum* ΔH (DSM 1053) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany), *Methanobacterium* sp. strain CB12 (35) was kindly provided by R. A.

* Corresponding author.

TABLE 1. Standard Gibbs free-energy changes ($\Delta G'$) at 25 and 55°C for reactions involved in propionate and butyrate oxidation in anaerobic systems (pH 7)[a]

Compound	Reaction	$\Delta G^{0'}$ (kJ/mol)	$\Delta G'_{55}$ (kJ/mol)
Butyrate	$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2 + H^+$	48.1	37.9
	$CH_3CH_2CH_2COO^- + 2HCO_3^- \rightarrow 2CH_3COO^- + 2HCOO^- + H^+$	45.5	36.1
Propionate	$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + 3H_2 + H^+$	76.1	62.3
	$CH_3CH_2COO^- + 2HCO_3^- \rightarrow CH_3COO^- + 3HCOO^- + H^+$	72.2	59.7
Acetate	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31.0	−34.7
Hydrogen	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−135.6	−122.5
Formate	$4HCOO^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^-$	−130.4	−118.9

[a] $\Delta G^{0'}$ values were taken from Thauer et al. (29) and Stams et al. (28); $\Delta G'_{55}$ values were calculated by using the Gibbs-Helmholtz equation with standard enthalpy values for compounds and $\Delta G^{0'}$.

Mah, University of California–Los Angeles, and the *Desulfotomaculum* sp. was isolated in our laboratory from a thermophilic sewage sludge reactor. All the bacteria were tested for the ability to grow on H_2 plus CO_2 and formate. Hydrogen-grown cultures of *Methanobacterium* sp. strain CB12 utilized formate without any lag phase.

Source of inoculum. The granules used were from a 5-liter thermophilic (55°C) UASB reactor originally inoculated with thermophilic digested manure and operated for 1 year with a hydraulic retention time of 4.5 h and a chemical oxygen demand removal efficiency of more than 90%. The reactor was fed with modified UASB medium (25) supplemented with 1 g of chemical oxygen demand each of acetate (15.6 mM), propionate (8.9 mM), and butyrate (6.2 mM) per liter.

Media and cultivation. A defined medium (3) was used for cultivation of the different hydrogen-utilizing bacteria. The gas phase was H_2-CO_2 (80:20) at 1 atm overpressure. For cultivation of the *Desulfotomaculum* sp., the medium was supplemented with yeast extract (1 g/liter), $NaSO_4$ (20 mM), and sodium acetate (2 mM). For cultivation of *Methanobacterium* sp. strain CB12 and *M. thermoautotrophicum* ΔH, sodium acetate (2 mM) was added. The temperature was 55°C.

Modified UASB medium (25) was used for cultivation of the granules, and the vials were inoculated at 55°C with shaking. In the vials containing the *Desulfotomaculum* sp., $NaSO_4$ was included in the medium at a final concentration of 5 mM. Samples of fresh wet granular sludge (2 or 5 ml) from the UASB reactor were distributed anaerobically under N_2 gas into serum vials. When H_2- and/or formate-utilizing bacteria were added, 1 ml of cultures in the late exponential growth phase was added to the vials. Each of the three hydrogen-utilizing bacteria had the same growth rate and lag phase whether grown in modified UASB medium (25) or defined medium (3). All experiments were run in triplicate.

The granules were acclimated in the incubator without added substrate for 1 h before sodium propionate or sodium butyrate was added to a final concentration of 8.9 or 6.2 mM, respectively (1 g of chemical oxygen demand per liter). Degradation of either butyrate or propionate was measured for 3 to 6 h before some of the granules were disintegrated with a tissue homogenizer (Thomas Scientific) under N_2 gas. After addition of H_2- and/or formate-utilizing bacteria, the degradation of VFA was monitored for another 3 to 6 h. To test for the effect of methanogens in the system, 2-bromoethanesulfonic acid (BES; 15 mM), a specific inhibitor of methanogenic bacteria (6, 36), was used to partially inhibit the methanogenic bacteria in the vials.

Statistical methods. The initial degradation or production rate of VFA was determined from the linear portion of the graphs of VFA produced per gram of volatile solids (VS) versus time by linear regression; all correlation coefficients were 0.9 or greater.

To test whether group means were equal, a one-way analysis of variance with multiple comparison was made by the methods of Tukey (27). The homogeneity of variance was tested by Bartlett's test (27). A 5% level of significance was always used.

A nonlinear regression (Marquardt-Levenberg algorithm) was used to estimate the best fit for the degradation rates of propionate and butyrate as a function of the hydrogen partial pressure.

Analytical methods. Hydrogen was quantified by gas chromatography with a reduction gas detector (Trace Analytical, Menlo Park, Calif.). The carrier gas was He, 10.5 to 11.5 ml/min, cleaned by a catalytic combustion filter (Trace Analytical). One milliliter of liquid from cultures was transferred with a syringe to 10-ml serum vials outgassed with hydrogen-free nitrogen. The vials were shaken to liberate the dissolved H_2, and a pressure-lock syringe was used for sampling (0.5 ml) (33). Methane and VFA were quantified by gas chromatography with flame ionization detection (3). The VS content was determined by standard procedures (8).

The effectiveness factor η, defined as the ratio of the degradation rate in disintegrated granules to the degradation rate in whole granules ($\eta = \text{rate}_{dis}/\text{rate}_{whole}$) (24), was used to compare degradation rates.

Calculation of hydrogen partial pressure for ΔG_{55}. The hydrogen partial pressure for $\Delta G_{55} = 0$ during degradation of propionate and butyrate was calculated according to the stoichiometries shown in Table 1.

RESULTS

Propionate as substrate. Figure 1 shows the time course of propionate degradation in the granules during the experimental period. The degradation rate was 1.16 μmol of propionate per min per g of VS. Methane was produced and acetate was degraded along with propionate. Disintegration of the granules decreased the degradation rate of propionate to 0.78 μmol/min/g of VS. The effectiveness factor was 0.67 (Table 2). Partial inhibition of the methanogens with BES resulted in a decrease in the degradation rate of propionate for both whole and disintegrated granules. However, addition of a hydrogen-consuming bacterium, a *Desulfotomaculum* sp., counteracted the inhibition by BES when the granules were whole. When the granules were disintegrated, the same degradation rate was observed as when only BES was added (Table 2). No accumulation of acetate was observed for whole or disintegrated granules during the degradation of propionate, but after addition of BES or BES

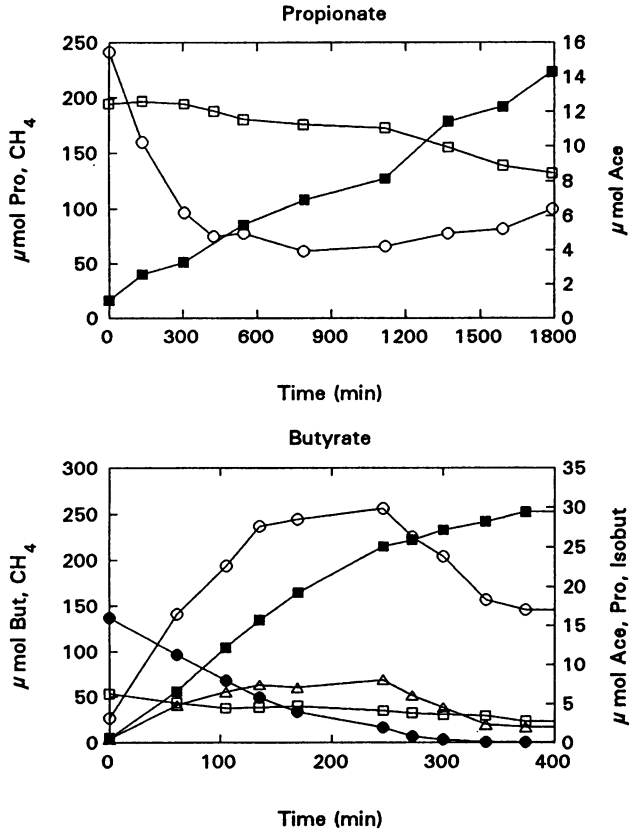

FIG. 1. Degradation or production of acetate (Ace), propionate (Pro), isobutyrate (Isobut), butyrate (But), and methane (CH_4) in whole granules with propionate or butyrate as the substrate. Symbols: ○, acetate; □, propionate; △, isobutyrate; ●, butyrate, ■, methane. Liquid volume, 22.5 ml.

and the *Desulfotomaculum* sp., acetate accumulated at 0.6 to 1.03 μmol/min/g of VS (Table 2).

Butyrate as substrate. Degradation of butyrate resulted in production of methane and a transient accumulation of acetate and isobutyrate, which were then degraded (Fig. 1). Degradation rates of 4.0 and 3.1 μmol of butyrate per min per g of VS were found for whole and disintegrated granules, respectively. The effectiveness factor was 0.77 (Table 3). Disintegration of the granules resulted in a transient accumulation of low concentrations of propionate and isobutyrate and a transient decrease in the acetate concentration. Addition of BES resulted in a decrease in the initial degradation rate of butyrate, but, as observed for the experiments with propionate degradation, no change in the rate was observed when a *Desulfotomaculum* sp. was also added (Table 3).

Hydrogen partial pressure during degradation of propionate and butyrate. The hydrogen partial pressure during oxidation of propionate in disintegrated granules was initially 1×10^{-2} atm but decreased quickly to a level of 5×10^{-4} atm, where it stayed for the rest of the experiment. The hydrogen partial pressure during start-up of the experiment was only slightly higher than the theoretical hydrogen partial pressure (P_{H_2}, approximately 4×10^{-3} atm), calculated to give a ΔG_{55} of 0. In the whole granules, the hydrogen partial pressure during oxidation of propionate was constant at 3×10^{-4} atm. The hydrogen partial pressure during degradation of butyrate in disintegrated granules was 2.5×10^{-2} atm at the beginning of the experiment but stayed at 9×10^{-3} atm during the rest of the experiment. The hydrogen partial pressure was sufficiently low to ensure that the ΔG_{55} was negative. The hydrogen partial pressure for whole granules degrading butyrate was constant at 8×10^{-3} atm.

Effect of addition of different hydrogenotrophic bacteria. Addition of either *M. thermoautotrophicum* ΔH, a hydrogen-utilizing methanogen that does not use formate; *Methanobacterium* sp. strain CB12, which is a hydrogen- and formate-utilizing methanogen; or a *Desulfotomaculum* sp., a sulfate-reducer that uses both hydrogen and formate, all led to an increase in the degradation rates of propionate and butyrate in disintegrated granules compared with the rates in intact granules or disintegrated granules without additions (Fig. 2). The increase was most pronounced after addition of the *Desulfotomaculum* sp. By measuring the hydrogen partial pressure in the cultures, a clear correlation was observed between the hydrogen partial pressure and the degradation rate of VFA, showing that increasing hydrogen partial pressure led to a decrease in the degradation rate of propionate and butyrate (Fig. 3).

DISCUSSION

Disintegration of the granules resulted in a decrease in the degradation rate for both butyrate and propionate, 20 and 30%, respectively, indicating that close contact between the microorganisms involved in the degradation was important. The decrease in the degradation rate observed after disintegration of the granules was probably due to disruption of microcolonies of syntrophic bacteria together with methanogens and hence an increase in hydrogen mass transfer resistance due to the increased distance between the hydrogen-producing and -utilizing bacteria. The decrease in the degradation rate after disintegration could also be due to

TABLE 2. Initial rates of acetate and propionate production or degradation in thermophilic granules with propionate as the substrate[a]

Addition(s)	Production/degradation rate (μmol/min/g of VS)				
	Acetate		Propionate		
	Whole granules	Disintegrated granules	Whole granules	Disintegrated granules	Effectiveness factor
Propionate	−0.42 (0.11)	−0.34 (0.05)	−1.16 (0.14)	−0.78 (0.05)	0.67 (0.08)
Propionate + BES	0.60 (0.04)	1.03 (0.08)	−0.87 (0.08)	−0.56 (0.09)	0.64 (0.12)
Propionate + BES + SRB[b] + SO_4^{2-}	0.70 (0.02)	0.93 (0.09)	−1.16 (0.02)	−0.56 (0.09)	0.48 (0.08)

[a] Values indicating degradation are preceded by a minus sign; other values indicate production. Values are arithmetic means; standard deviations are shown in parentheses ($n = 3$).
[b] SRB, a *Desulfotomaculum* sp.

TABLE 3. Initial rates of acetate, propionate, isobutyrate, and butyrate production or degradation in thermophilic granules with butyrate as the substrate[a]

Addition(s)	Production/degradation rate (μmol/min/g of VS)								
	Acetate		Propionate		Isobutyrate		Butyrate		
	Whole granules	Disintegrated granules	Whole granules	Disintegrated granules	Whole granules	Disintegrated granules	Whole granules	Disintegrated granules	Effectiveness factor
Butyrate	1.07 (0.14)	−0.47 (0.06)	−0.10 (0.01)	0.09 (0.03)	0.41 (0.07)	1.1 (0.4)	−4.0 (0.1)	−3.1 (0.3)	0.77 (0.08)
Butyrate + BES	4.55 (0.86)	ND[b]	−0.066 (0.02)	ND	0.40 (0.06)	ND	−3.2 (0.5)	ND	ND
Butyrate + BES + SRB + SO_4^{2-}	5.57 (0.82)	ND	−0.048 (0.009)	ND	1.0 (0.8)	ND	−4.1 (0.4)	ND	ND

[a] See Table 2, footnotes *a* and *b*.
[b] ND, not determined.

damage to the syntrophic bacteria occurring during disintegration. However, disintegrating the granules had no influence on the degradation of acetate, as previously shown for granules from a UASB reactor fed with acetate as the substrate (24), and the same propionate and butyrate degradation rates could be obtained after addition of hydrogen-utilizing bacteria, indicating that the mass transfer resistance was the factor of importance. Addition of BES during both propionate and butyrate degradation resulted in a partial inhibition of methanogenesis in both whole and disintegrated granules; the degradation rates of propionate and butyrate decreased 25 and 20%, respectively. However, after addition of a *Desulfotomaculum* sp., propionate and butyrate were degraded as fast as in whole granules. This indicated that (i) significant propionate and butyrate oxidation only occurred if hydrogen or formate was effectively removed by the hydrogen- and/or formate-utilizing bacteria and (ii) acetate at the observed concentrations (up to 12 mM) had no influence on the catabolism of propionate and butyrate.

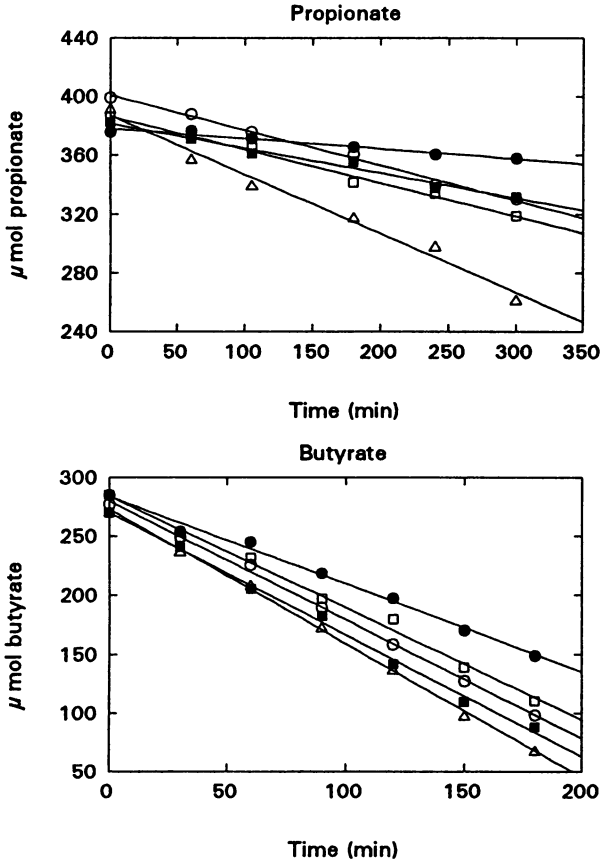

FIG. 2. Degradation of propionate and butyrate in disintegrated granules after addition of hydrogenotrophic bacteria as a function of time with propionate or butyrate as the substrate. Symbols: ●, disintegrated granules. Also represented are disintegrated granules with *Methanobacterium thermoautotrophicum* ΔH (○), *Methanobacterium* sp. strain CB12 (□), and the *Desulfotomaculum* sp. (△); ■, whole granules.

The hydrogen partial pressure measured during degradation of propionate and butyrate by whole granules, 3×10^{-4} and 8×10^{-3} atm, respectively, gives information only about the hydrogen partial pressure in the bulk-fluid phase, not inside the granules. By disintegrating the granules, the definitive partial pressure of hydrogen can be estimated more accurately, and consequently the influence of hydrogen on degradation can be better evaluated. During degradation of propionate and butyrate in disintegrated granules, the hydrogen partial pressure was below the hydrogen partial pressure for $\Delta G_{55} = 0$, calculated from the actual concentrations of substrates and products. When butyrate was the substrate, the hydrogen partial pressure was ca. 9×10^{-3} atm. The ΔG_{55} value for propionate oxidation in disintegrated granules with butyrate as the substrate, when calculated from the actual concentrations of propionate, acetate, and hydrogen, was positive (ca. 21 kJ/mol). This indicated that propionate oxidation was unfavorable under these conditions. In accordance with this, a concomitant accumulation of propionate was observed during degradation of butyrate in disintegrated granules. The source of this propionate was unknown, but it could be the result of a back-reaction in which acetate and hydrogen are condensed into propionate under conditions of high hydrogen concentrations, or it could indicate that propionate degradation proceeded at a steady rate in all the samples but that propionate accumulated only when its degradation was inhibited by an increased hydrogen concentration.

Three different bacteria with different abilities to utilize either hydrogen and/or formate and with different kinetics (K_s and threshold values) were used. The sulfate-reducing bacterium used, a *Desulfotomaculum* sp. which uses H_2 and formate, has a very low K_s (ca. 5×10^{-5} atm) and threshold value (ca. 10^{-7} atm) for hydrogen (unpublished data) compared with the values for the methanogens used. *Methanobacterium* sp. strain CB12 (uses H_2 and formate) and *Methanobacterium thermoautotrophicum* ΔH (uses only H_2) have approximately the same K_s (2×10^{-4} atm) and threshold values (5×10^{-5} atm) for hydrogen (1, 17, 23; unpublished

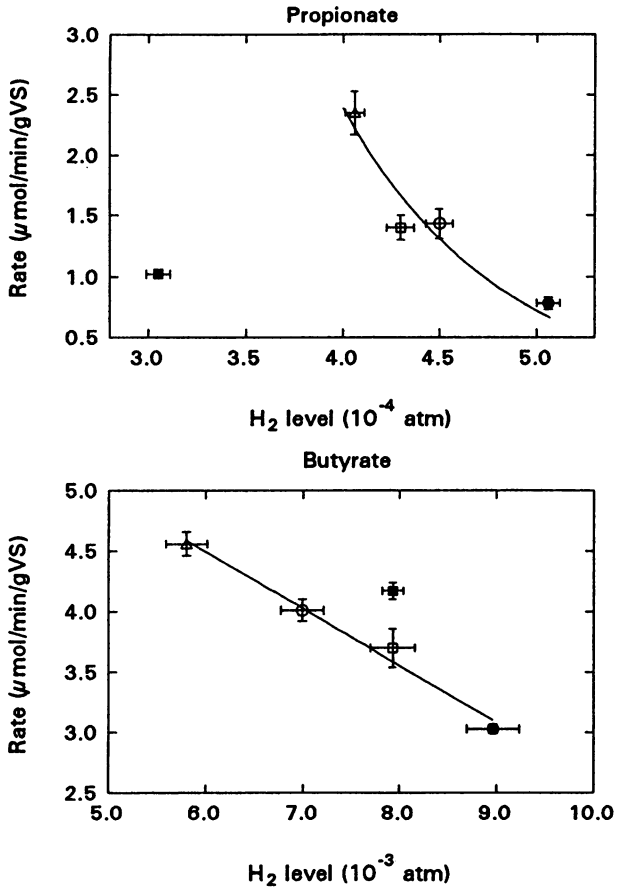

FIG. 3. Degradation rates of propionate and butyrate as a function of hydrogen partial pressure with propionate or butyrate as the substrate. Symbols: ●, disintegrated granules. Also represented are disintegrated granules with *Methanobacterium thermoautotrophicum* ΔH (○), *Methanobacterium* sp. strain CB12 (□), and the *Desulfotomaculum* sp. (△); ■, whole granules. Best-fit lines for degradation rates were exp [(*a* × hydrogen partial pressure) + *b*] for propionate and (*a* × hydrogen partial pressure) + *b* for butyrate. Error bars show standard deviations for trials in triplicate.

data). Addition of these hydrogen-consuming bacteria to disintegrated granules resulted in an increased degradation rate for propionate and butyrate compared with the rates in vials with no bacteria added. For both propionate and butyrate, the stimulatory effect on the degradation rate was highest after addition of the *Desulfotomaculum* strain, causing increases of 300 and 150%, respectively. These findings are in accordance with the general observation that syntrophic cocultures grow faster when sulfate reducers instead of methanogens are used as hydrogen scavengers (4, 10, 19, 20). In our experiments, no significant differences in the effects of adding *Methanobacterium* sp. strain CB12, which uses both H_2 and formate, or *M. thermoautotrophicum* ΔH, which uses only H_2, were observed, indicating that interspecies formate transport was not essential for the observed effect on the degradation rate. The increase in the degradation rates in the disintegrated granules was 180 and 125% for propionate and butyrate, respectively.

Boone et al. (5) proposed that interspecies formate transfer was the predominant mechanism of syntrophy. This conclusion was made from diffusion calculations showing that hydrogen could not diffuse rapidly enough to dispersed methanogenic cells in digestors with a cell concentration of 10^7 cells per ml (giving an average distance of 46 μm between cells) to account for the rate of methane synthesis but formate could. This result was based on the assumption that the average distance between microbes was high (more than 10 μm), so that the presence of H_2-producing microbes did not affect hydrogen uptake except as they affected the concentration in the bulk solution. However, in granules, the cell concentrations are much higher than in dispersed cultures, and they consist of microcolonies (12, 13) in which the distances between the syntrophic bacteria and the hydrogen-utilizing bacteria are very small (less than 5 μm). Therefore, in a system like granular sludge, the diffusion of hydrogen can be great enough to account for the methane production, and consequently, interspecies hydrogen transfer can be the predominant mechanism in the syntrophic degradation. Dispersed thermophilic syntrophic cocultures degrading propionate (28) or acetate (15) in which formate seems to be of no importance for interspecies electron transfer have also been described in the literature.

By monitoring the hydrogen partial pressure in the liquid, a clear correlation was found between the hydrogen partial pressure and the degradation rate of propionate and butyrate in disintegrated granules, showing a decrease in the degradation rate with increasing hydrogen partial pressures. This experimental result is in agreement with theoretical thermodynamic considerations, showing that the degradation rate of an organic substrate in syntrophic cocultures is dependent on the ability of the hydrogen-utilizing bacteria to utilize low concentrations of hydrogen. The formate concentration in our systems was below the detection limit of our analytical method (1 mM). However, from thermodynamic considerations, it cannot be excluded that the same type of correlation exists between the formate concentration and the degradation rates of propionate and butyrate. Our results clearly indicated a close dependency between the propionate- or butyrate-degrading bacteria and the hydrogen-consuming methanogens. If the hydrogenotrophic bacteria were able to utilize hydrogen fast enough and thereby lower the hydrogen partial pressure, the oxidizing bacteria had a greater capacity for degradation.

ACKNOWLEDGMENTS

We thank Carl Otto Frølund Jørgensen for excellent technical assistance.

This work was supported with funds from the Center for Process Biotechnology to Jens Ejbye Schmidt and from the Nordic Ministerial Council to Birgitte Kiær Ahring.

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